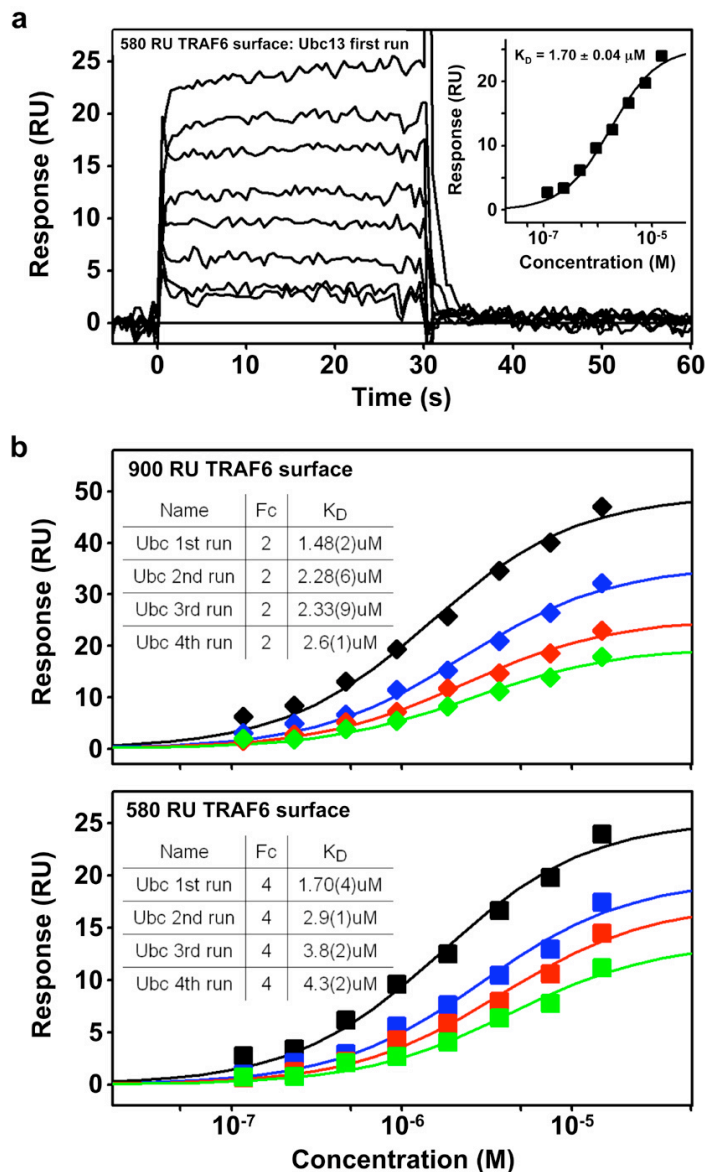
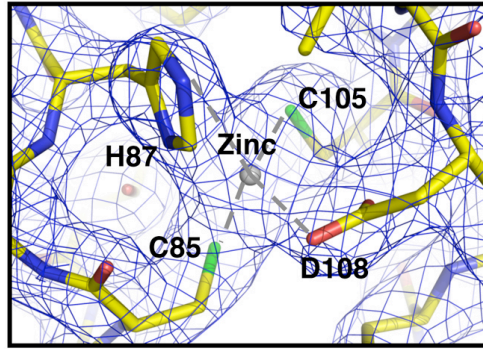


E2 interaction and dimerization in the crystal structure of TRAF6

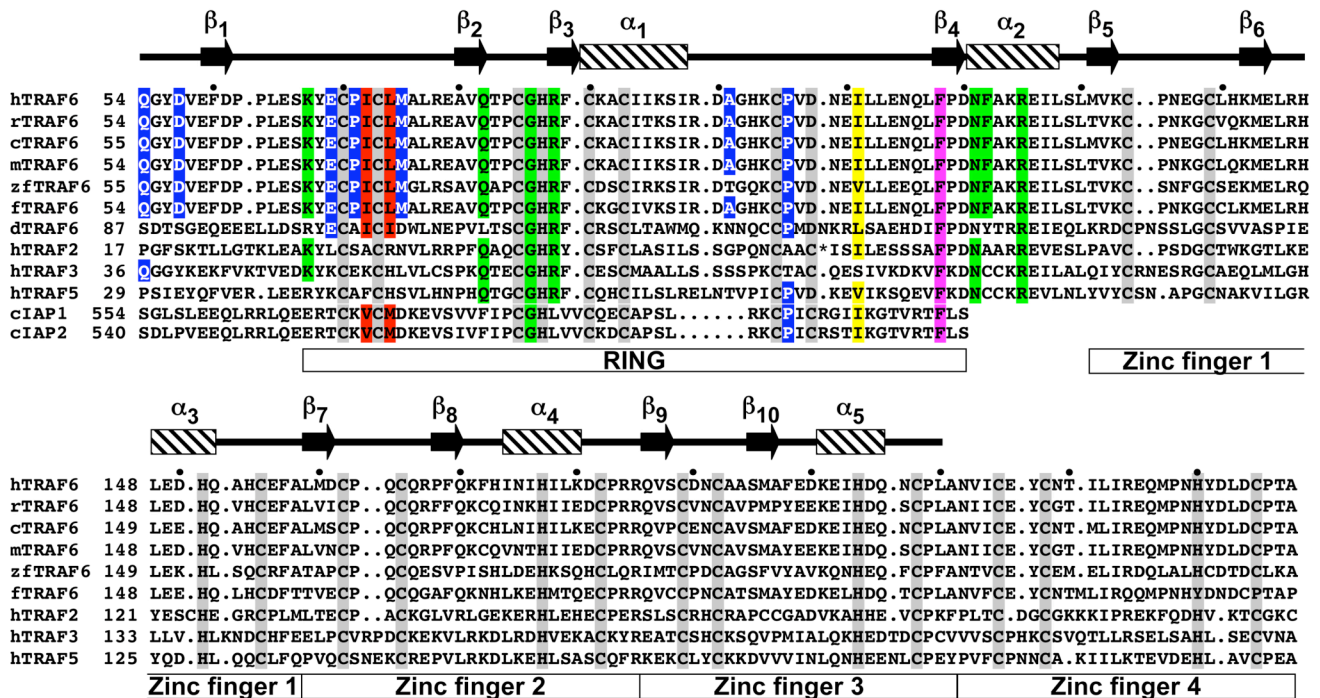
Qian Yin, Su-Chang Lin, Betty Lamothe, Miao Lu, Yu-Chih Lo, Gregory Hura, Lixin Zheng, Rebecca L. Rich, Alejandro D. Campos, David G. Myszka, Michael J. Lenardo, Bryant G. Darnay and Hao Wu



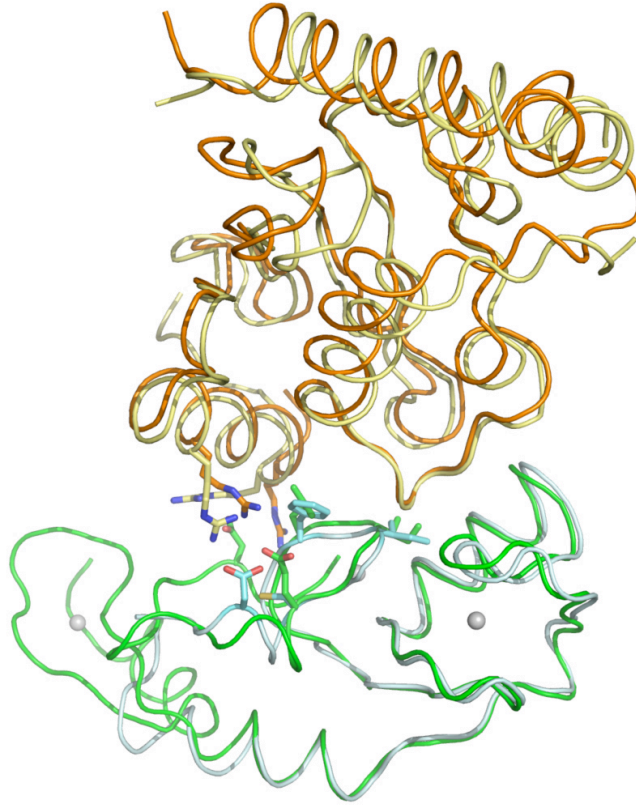
Supplementary Figure 1. Quantitative measurement on the interaction between TRAF6 and Ubc13 using SPR. **(a)** Binding of Ubc13 at a series of concentrations to the 580 RU surface coupled with TRAF6 RZ₁₂₃. The responses at 10-20 seconds were plotted against Ubc13 concentration and fit to a simple binding isotherm to obtain affinity information. **(b)** To establish the reproducibility of the SPR measurements, the experiment was repeated another three times at both 900 and 580 RU surfaces, which showed a gradual loss in response intensity over time. The decay in intensity indicates that the TRAF6 surfaces were losing appreciable activity during the time required for these measurements. Black: first run; blue: second run; red: third run; green: fourth run. The fitted dissociation constants were shown.



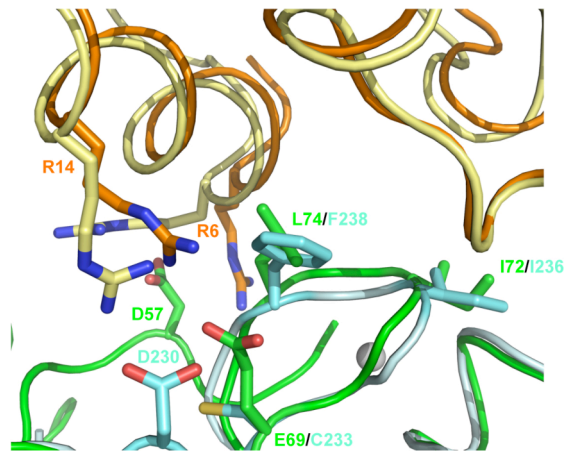
Supplementary Figure 2. 2Fo-Fc Electron density map (contoured at 1.0 σ) at one of the zinc coordinating regions of the TRAF6 RZ₁₂₃ structure



Supplementary Figure 3. Sequence alignment of TRAF6 from different species and with TRAF2, TRAF3, TRAF5, cIAP1 and cIAP2. Domain boundaries and secondary structures are labeled. Zinc-coordinating residues in the RING and zinc fingers 1-4 domains are shaded in gray. Residues at the Ubc13 interface are highlighted in red for those that bury more than 60 \AA^2 surface area and in blue for those with surface area burials of 20-60 \AA^2 . Residues at the dimerization interface are highlighted in magenta for those that bury more than 100 \AA^2 surface area and in green for those with surface area burials of 40-100 \AA^2 . The conserved residue at the modeled tetramerization interface is highlighted in yellow. "*" in the TRAF2 sequence represents an insertion of "VHEGIYEEG". h: human; r: rat; c: cow; m: mouse; zf: zebra fish; f: fowl; d: drosophila.



Supplementary Figure 4. Superposition of the TRAF6/Ubc13 complex with the CHIP/Ubc13 complex. Green: TRAF6; Cyan: CHIP; orange: Ubc13 in the TRAF6 complex; yellow: Ubc13 in the CHIP complex.



Supplementary Figure 5. Details in the superposition of the TRAF6/Ubc13 complex with the CHIP/Ubc13 complex. Green: TRAF6; Cyan: CHIP; orange: Ubc13 in the TRAF6 complex; yellow: Ubc13 in the CHIP complex.

Supplementary Methods

Yeast two-hybrid analysis. We received the *S. cerevisiae* strain AH109 as a generous gift from Dr. Shrikanth Reddy (UTMDACC). We co-transformed yeast cells with the indicated genes in pGAD-T7 and pGBK-T7 based plasmids using the lithium acetate method as stated by the manufacturer (Clontech). Following double selection on synthetic-dextrose (SD) lacking Trp and Leu (SD-Trp-Leu), we picked at least three separate colonies and spotted them onto both SD-Trp-Leu and SD-Trp-Leu-His (with 5 mM 1,2,4-amino triazole) plates. We incubated the plates at 30 °C until growth appeared on the master plate (SD-Trp-Leu), typically within 2-3 days, and determined positive interactions as equivalent growth of colonies on SD-Trp-Leu-His plates.

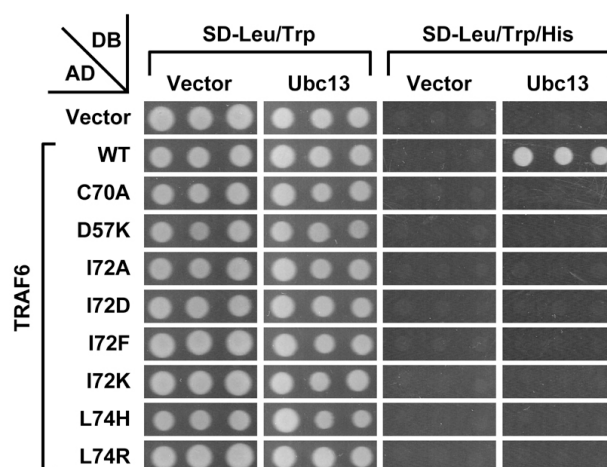
Transfection, reporter gene assays, and retroviral infection. We Transfected HEK293 cells, produced retroviral supernatants and performed retroviral infection essentially as described previously^{1,2}.

Immunoprecipitation, western blotting and in vitro kinase assay. We left the cells unstimulated or stimulated as indicated in the figure legends and washed them twice with PBS. Depending on the experiment, we lysed the cells with lysis buffer and processed them for immunoprecipitation, western blotting, and in vitro kinase assay as previously described^{1,2}.

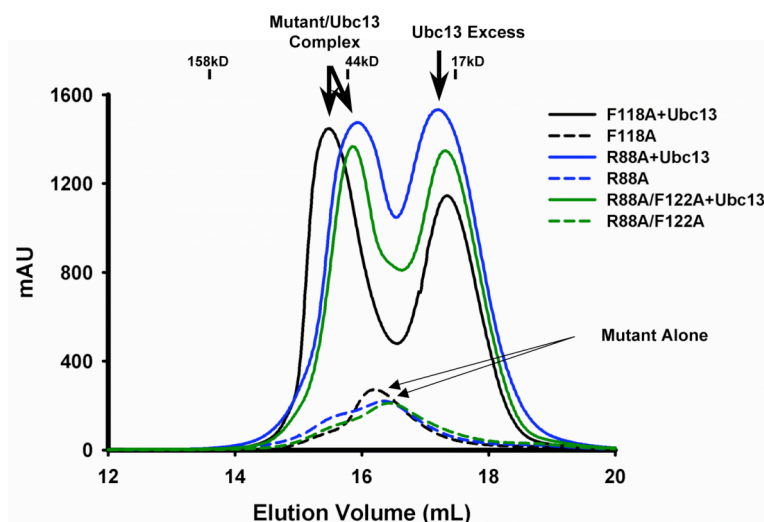
FRET assay. We fused wild type or F118A mutant mouse TRAF6-coding sequences at the N-terminus of Cerulean or Venus coding sequences. We transiently transfected the expression constructs into BJAB lymphoma cells by electroporation, in which we re-suspended 4 million cells in 400 µl of complete RPMI medium in a 0.4-cm cuvette, with a total of 20 µg DNA. We electroporated the cells at 260 V, 1050 mF, and 720 W, using a BTX-600 machine. We cultured the cells for 24~36 hours before analyzing them using flow-cytometry with A BD LSR II system. We examined fusion-protein expression using excitation lasers of 407 nm /488 nm and measuring emissions at 470 nm /550 nm for Cerulean and Venus respectively. We detected FRET at a 550 /50 nm band-pass filter by excitation at 407nm.

Confocal microscopy of TRAF6 distribution. We incubated BJAB cells (ATCC) with 10 µg ml⁻¹ of lipopolysaccharides (LPS) (Sigma) for 2 hours and subjected them to the following processes at room temperature. We washed the cells with PBS twice, re-suspended them in 1 ml of 1 % (v/v) paraformaldehyde/PBS for 15 minutes, and washed them once with PBS. We used Cytospin to transfer the fixed cells onto poly lysine-coated microscope slides. We permeabilized the mounted cells with 0.2 % (v/v) Triton X-100/PBS for 30 minutes, blocked them by 1 % (w/v) BSA in PBS for 1 hour, and stained them with 1 µg ml⁻¹ of rabbit anti-TRAF6 (ZyMED) for 1 hour. We washed the cells 3 times with PBS and stained them with Alexa Fluor 568-goat-anti-rabbit IgG (Molecular Probes) (1:500) and 1 µM Hoechst 33342 (Molecular Probes) for 1 hour. We then rinsed the slides 3 times against PBS and mounted them with cover slides. We used a Leica Sp1 confocal microscope to scan images of TRAF6 and nucleus staining and the Imaris 6.1 software to analyze at least 10 40-x-images for each treatment.

1. Lamothe, B. et al. Site-specific Lys-63-linked tumor necrosis factor receptor-associated factor 6 auto-ubiquitination is a critical determinant of I kappa B kinase activation. *J Biol Chem* **282**, 4102-12 (2007).
2. Besse, A. et al. TAK1-dependent signaling requires functional interaction with TAB2/TAB3. *J Biol Chem* **282**, 3918-28 (2007).



Supplementary Figure 6. Yeast two hybrid experiments of Ubc13 binding mutants of full-length TRAF6. C70 is a zinc-coordinating residue in TRAF6 RING and C70A is a negative control for Ubc13 interaction.



Supplementary Figure 7. The dimerization mutants of TRAF6 RZ₁₂₃ F118A, R88A and R88A/F122A retained their interactions with Ubc13 on gel filtration chromatography. Approximate elution positions of molecular weight standards are shown.